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13. ABSTRACT (Maximum 200 Words)

A limitation of specific adoptive immunotherapy this therapy is the poor survival and tumor localization of activated T cells after infusion into a host. A contributor to these problems is the appendage-bearing, adhesive form of activated T cells, which renders them poorly suited to circulate, and likely to embolize in microvasculature. We hypothesized that transient inhibition of T cell appendage formation and adhesiveness, induced just before infusion, will improve the survival and circulation of adoptively transfered T cells. We found previously that pretreatment of activated T cells with the myosin light-chain kinase inhibitor ML-7 renders the cells temporarily smooth and nonadhesive. In this project we developed an ML-7 pretreatment protocol which allowed activated T cells to recover normal adhesion, motility, cytotoxicity, and proliferation within 24 hr. We then found that this protocol decreased by eightfold the percentage of infused cells trapped in the lung and increased fourfold the homing of ErbB2-specific T cells to the ErbB2+ murine mammary tumor D2F2/E2. Homing of infused T cells to peripheral lymph nodes was also increased by a factor of 1.5. When tested in a D2F2/E2 immunotherapy model, the ML-7 pretreatment of T cells was found neither to enhance nor reduce the tumor-delaying effects of T cell infusion in D2F2/E2-bearing mice. The results constitute the first proof that cytoskeletal alteration of T cells can improve their trafficking behavior after adoptive transfer. On the basis of our initial test, though it appears that this strategy alone is enough to enhance adoptive immunotherapy. The ML-7 depolarization strategy is easily combined with other new methods of improved immunotherapy, such as transduction of T cells with cytokines and survival genes, and such combinations should be tested in the future.

14. SUBJECT TERMS
Tumor immunology, adoptive immunotherapy, lymphocyte traffic, homing, cytoskeleton, motility

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INTRODUCTION

Relapse after surgery and chemotherapy is a major cause of therapeutic failure in breast cancer patients. Vaccination against tumor antigens is a strategy which holds great promise for preventing recurrence, but it requires an intact immune system [1]. Immunotherapy by adoptive transfer of activated T cells is potentially an excellent way to prevent local and metastatic tumor growth during the period of immunological depletion which follows intensive radiotherapy and chemotherapy [2]. Patient T cells are stimulated in vitro to a peak of population size and anti-tumor activity and infused back into the patient. A serious limitation on adoptive immunotherapy is the poor ability of the infused T cells to circulate properly. A contributing factor to this problem is the morphological and behavioral phenotype of the cells. T cells normally circulate in a state of rest or early activation, in which they are round, smooth, and nonadhesive. Activation in vitro pushes them into a polarized, spiky, adhesive and highly motile form which they never normally achieve until after extravasation. When infused in this polarized state, T cells tend to clump together and to lodge in microvasculature. Many are trapped, embolized, and damaged during their initial passages through pulmonary microvasculature, and are later destroyed in the liver and spleen. Relatively few transferred cells survive to enter tumor masses and secondary lymphoid organs [3]. The polarized, adhesive phenotype of activated T cells is created by the actin-myosin cytoskeleton, which, in activated T cells, is programmed to produces appendages and to hold adhesion receptors in an activated state [4;5;5]. We have found previously that activated T cells can be rendered temporarily spherical, smooth, and nonadhesive in vitro by treatment with pharmacological inhibitors of myosin function. We hypothesized that pretreatment of activated T cells with myosin inhibitors prior to infusion will allow the cells to avoid initial trapping in lung microvasculature and circulate widely before regaining the polarized, tissue-invading phenotype. We further hypothesized that this would in turn increase localization of the infused T cells into tumors and lymph nodes, and thus augment immunotherapeutic effect. During the course of this project we tested these hypothess in a murine model of breast cancer.

BODY:

Note: The Aims and Tasks referred to below are not those of the original Statement of Work but rather the revised Statement of Work approved January 2002.

Aim 1: To test the hypothesis that temporary depolarization of activated T cells by pharmacological myosin inhibitors will increase the percentage of cells localizing in tumor masses and secondary lymphoid organs after adoptive transfer.

Task1: "Determine effects of myosin-inhibiting drugs on *in vivo* T cell localization."
Task1 a and b: . Immunize mice; obtain and specifically expand ErbB2-specific T cell populations.
These tasks were completed during the first year of work and summarized in the first annual report. The results will be summarized in some detail, though, because they are necessary for understanding more recent work.

Task 1 a and b RESULTS:

The procedure for producing murine T cells specific for human ErbB2 specific was developed by a colleague, Wei-Zen Wei, who provided all required cell lines The tumor used in this study was D2F2/E2, a mammary adenocarcinoma which arose from a spontaneous mammary hyperplasia of a BALB/C mouse and which was stably transfected to express full-length human ErbB2 [6]. Syngeneic BALB/c mice received bilateral s.c. flank injections of 5 X 10^5 irradiated D2F2/E2 cells. This was repeated 4 weeks later, and after an additional 4 weeks draining lymph nodes were excised. ErbB2-specific lymphocytes from these nodes were specifically expanded by culture on irradiated cells from a syngeneic fibroblastoid cell line engineered to stably express human ErbB2 as well as K(d), IA(d)$\alpha\beta$, and the costimulator B7.1 [7]. This stimulator line will be referred to as "3T3/E2". The T cells were thinned and fed every 2 days with DME containing 10%FCS, 5U IL-2/ml and 10 ng/ml IL-7, and restimulated with irradiated 3T3/E2 every 14-20 days.

After two rounds of stimulation, the lymphocyte population reached a stable phenotype of >98% CD3+, of which typically 55-60% were CD4+, and 45-50% CD8+. After 3 simulations, lymph node cells from 12 mice could be expanded to up to 1.2 X 10^8 cells. They were used in experiments from 7 - 10 days after their last stimulation. At this point the cells typically exerted approximately 50% specific cytotoxicity against D2F2E2 at a an E:T ratio of 50:1, 35% at 25:1, and 10% at ratio of 10:1, as determined by chromium release assay (not shown). Cytotoxicity vs. the unrelated syngeneic mammary tumor 410.4 was <5%.

Task 1c. Determine effects of myosin inhibiting drugs on localization of T cells in mice bearing subcutaneous tumors.

Task 1c METHODS, part 1

Depolarization strategy. In the ErbB2-specific T cell cultures mentioned above, greater than 90% of cells displayed polarized elongated form, with complex anterior and posterior appendages. They were highly adhesive and prone to form clumps. Both appendage formation and integrin function are known to be dependent on an intact and normally functioning cytoskeleton. We found previously that chemical inhibitors of the contractile action of myosin can temporarily abolish appendages and adhesive function in activated lymphocytes and polarized leukemia cells. For this project we developed the a depolarization protocol for the ErbB2-specific T cell cultures. The cells were treated 20 min. with the myosin light-chain kinase inhibitor ML-7 (Calbiochem, 75 uM) followed by three washes. This treatment rendered 100% of the cells spherical, appendage free, and nonadhesive for a period of 30 - 60 min at 37°C. There was no loss of viability. Recovery of polarity, adhesiveness, and motility was complete by 6 hr after treatment, and cytotoxicity, and antigen-stimulated proliferation were fully recovered by 24 hr. **(Figs. 1 and 2 of Appendix, which are reproduced for convenience as Figs 1 and 2 below).**

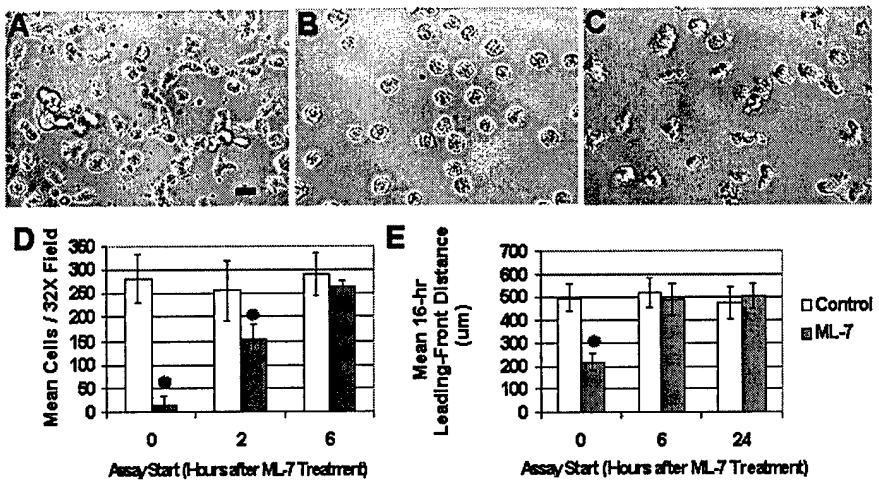

Figure 1. Effects of 20-min. treatment with MLCK inhibitor ML-7 (75 uM) upon morphology, adhesion, and motility of activated anti -D2F2/E2 T cells. **A-C.** Alterations in morphology. **A.** Prior to treatment. Note polarized morphology and frequent cell aggregates. **B.** Thirty min. after treatment and drug wash-out. Note smooth, spherical morphology and lack of aggregation. **C.** Six hours after drug wash out. Bar = 10 um. **D.** Changes in ability of anti-D2F2/E2 T cells to adhere to fibronectin over a period of 1 hour. **E.** Changes in spontaneous 16-hr. migration into three-dimensional gels of Type I collagen. In **D.** and **E.**, X-axis indicates number of hours elapsed between drug wash-out and start of assay and asterisks indicate significant differences between control and ML-7 values ($p<0.05$).

Lymphocyte localization experiments. Subcutaneous s.c. D2F2/E2 tumors were allowed to grow to approx. 10 mm diameter (0.4 - 0.6 g), a process which took 3-4 weeks.

The green fluorescent tracking dye CFSE ("Cell Tracker", Molecular Probes) was used to label ErbB2-specific T cells before infusion into these tumor-bearing mice. In pilot studies it was determined that a concentration of 1 uM CFSE conferred sufficient fluorescent brightness to track the cells for at least 48 hr while not reducing the ability of the cells to exert cytotoxicity or to migrate into collagen matrix in vitro.

T cells were treated with ML-7 or DMSO vehicle control and washed as described above, and then infused into D2F2/E2-bearing mice via tail vein, 2×10^7 cells/0.5 ml HBSS per mouse. At 30 min, 2 hr, 24 hr, and 48 hr, tissues were harvested, weighed, and dispersed into single cell suspension either by physical disruption (for lymph nodes and spleen) or by a standardized protocol of digestion with Liberase (a commercial collagenase blend), elastase, and DNAse. The resulting single-cell suspensions were fixed and measured aliquots were analyzed by flow cytometry for both frequency and absolute numbers of viable CFSE-labeled lymphoid cells. In some experiments, tissues were not dispersed but frozen for histological examination of labeled cell distribution.

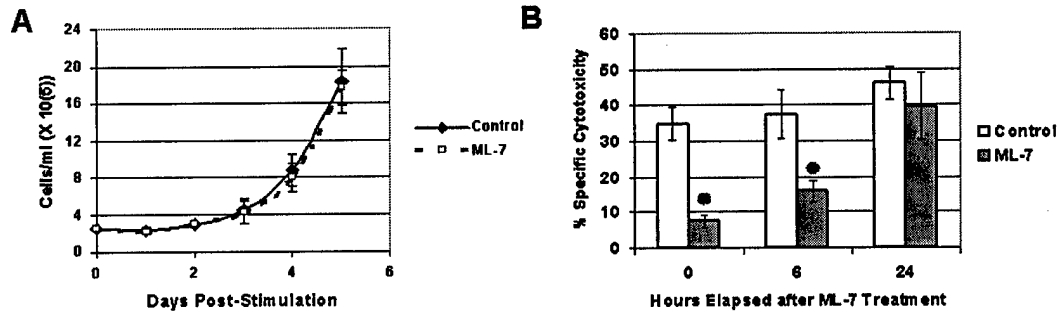

Figure 2. Recovery of anti-D2F2/E2 T cell function after transient depolarization by ML-7. A. Proliferation during incubation with irradiated stimulator cells. B. Cytotoxicity vs. target tumor cells at E:T = 25:1. Asterisks = significantly different from control $p < 0.05$.

Task 1c RESULTS, part 1

As described in detail in submitted manuscript (Appendix), prompt and transient localization of labeled cells is an indicator of trapping in the pulmonary microvasculature. We found that trapping of ErbB2-specific T cells in the lung was reduced eight-fold by pretreatment with ML-7 ($p \leq 0.05$) **(Fig 3A-C of Appendix)**. Localization of T cells in s.c. tumors was increased by > 4-fold over controls at both 24 and 48 hr. **(Figs 4A-C of Appendix, which are reproduced for convenience as Figs. 3A-C, below)**. This represented approximately 2% of cells infused per tumor. Localization determined by cytometry was found to reflect true extravasation of labeled cells into tumor parenchyma, rather than trapping in vascuature or the ingestion of labeled debris by phagocytic cells. In histological sections, CFSE fluorescence was found in intact cells of lymphoid morphology which were located well within parenchymal areas of tumor tissue **(Figs 4D,E of Appendix, reproduced for convenience as Figs 3 D,E, below)**.

Localization in lymph nodes also showed a small but significant increase for the ML-7-treated populations **(Fig 3D of Appendix)**. The results are consistent with the concept that transient depolarization of the activated T cells reduced their trapping and damage in the lung and thus increased the number of cells able to circulate and eventually reach tumor sites.

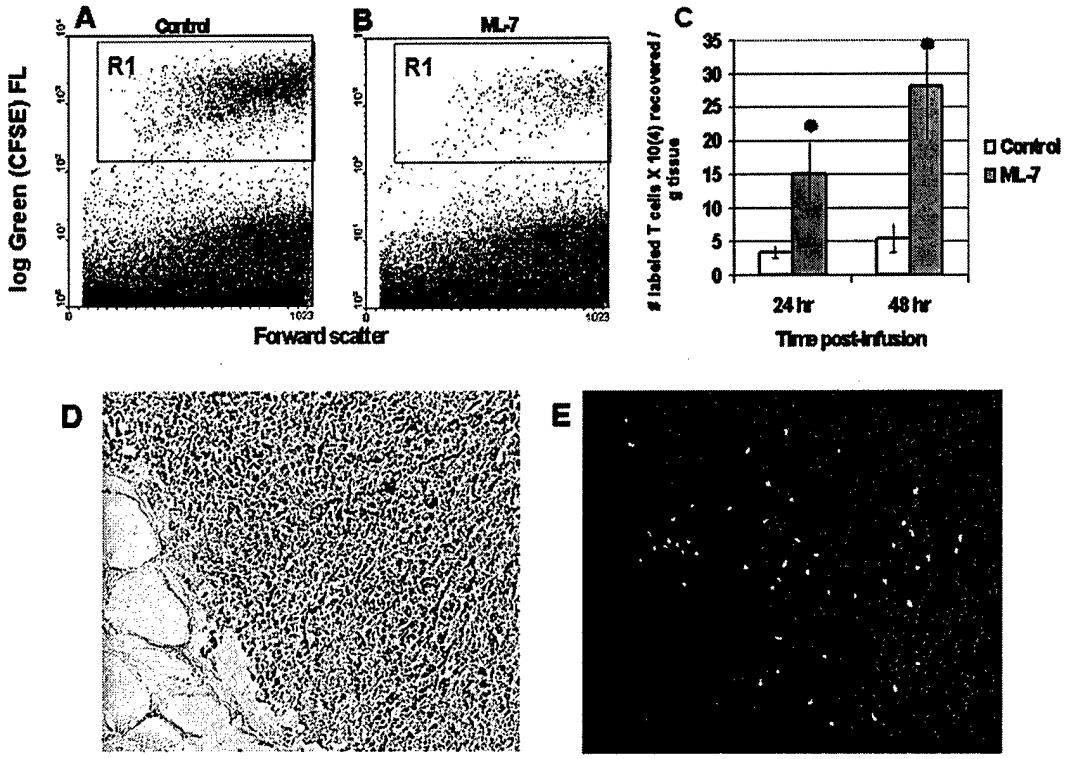

Figure 3. Localization of CFSE-labeled T cells in s.c. D2F2/E2 tumors. **A and B.** Typical flow cytometric analyses of single-cell suspensions of tumor at 24 hr after infusion of 2 X 10^7 labeled cells. **A.** Control-treated T cells. **B.** T cells treated 20 min with 75 uM ML-7. R1 = region of high fluorescence determined to be unique to viable labeled T cells. **C.** Data summary from entire series of experiments. Asterisks = significantly different from control $p < 0.05$. **D. and E.** Typical frozen section of tumor showing that fluorescent events detected by flow cytometry represent intact CFSE-labeled cells which have extravasated into tumor parenchyma. **D.** Phase contrast image. **E.** Green fluorescence image of same section. Tumor parenchyma is still visible by faint autofluorescence. Bar = 100 uM.

To complete this task, we next determined whether the ML-7-induced increase in T cell localization in tumors was sufficient to produce a measurable improvement in therapeutic effect.

Task 1c METHODS, part 2.

Effector cells. ErbB2-specific T cells for adoptive immunotherapy were grown as described above.

Tumors. D2F2/E2 tumors were initiated s.c. in the flanks of mice, bilaterally, as described above, but were allowed to grow only for 14 days before the start of an experiment, at which point they had become barely palpable. This time point was selected because our hypothesis requires the presence of a mass of tumor cells for the T cells to infiltrate; and because a relatively small tumor burden is more likely to respond to therapy.

In the pilot study, designed to establish therapeutic protocol, mice received i.v. tail vein infusions of 0.5 ml HBSS either alone or containing untreated T cells at doses ranging from 1 to 5 X 10^7 cells per mouse. For experiments determining the effects of ML-7 on therapy, infusions of 3 X 10^7 were given. Mice were divided randomly into three groups: "SALINE" mice received a tail-vein i.v. infusion of 0.5 ml HBSS. "CELLS + VEH" mice received 0.5 ml HBSS containing T cells which had been treated with vehicle (DMSO) for 20 min, washed 3X, and suspended in HBSS. "CELLS + ML-7" mice received T cells which had been treated 20 min with 75 uM ML-7, washed three times and resuspended in HBSS. Beginning at 7 days post-treatment, tumors were measured with calipers at weekly intervals. Two perpendicular measurements of diameter were made and the average value for each tumor will be referred to as "mean tumor diameter" [6]. Three experiments were performed, with 2 mice in each experimental condition in each experiment. ANOVA (Instat, Graphpad) was used to detect significant treatment-induced differences in mean tumor diameter. When differences were found, ANOVA was followed by paired t test with Bonnferoni correction for multiple comparisons.

Task 1c RESULTS, part 2

1.PILOT STUDY: OPTIMIZATION OF ADOPTIVE THERAPY PROCEDURE. To establish a protocol, variable numbers of ErbB2-specific T cells were infused i.v. into tumor-bearing mice. The cells began to produce an effect -- significantly reduced tumor growth -- at 2 X 10^7 per infusion (**not shown**). Maximal effect was achieved with 3 X 10^7 and did not increase significantly when 4 or 5 X 10^7 cells were administered. Even with maximal effect tumors were not eradicated or permanently arrested. Relative to controls, tumors size was 30% at 7 days, 50% at 14 days, and not significantly different at 21 and 28 days.

The results of the pilot study show that infusions of 3 X 10^7 ErbB2-specific T cells produce a significant but incomplete and temporary reduction in the development of s.c. D2F2/E2 tumors. This is an ideal situation for determining whether cytoskeletal alteration of the T cells produces greater or longer-lasting improvement in immunotherpeutic effect.

2. IMMUNOTHERAPEUTIC EFFECTS OF INCREASED TUMOR LOCALIZATION OF ML-7-TREATED T CELLS. As expected from the pilot study, a single infusion of T cells pretreated only with DMSO vehicle significantly reduced the growth of s.c. D2F2/E2 tumors for the first two weeks after treatment, relative to infusion of HBSS only (SALINE vs. CELLS + VEH in **Fig. 5 of Appendix, reproduced for convenience below as Fig. 4.).** Also consistent with the pilot study, cell infusion had no significant effect on longer-term outcome. By 4 weeks post treatment all tumors had grown to a size that required euthenasia of host mice in both SALINE and CELLS + VEH conditions **(Fig. 4, below).** .

Surprisingly, despite their greater tumor localization, the ML-7-pretreated cells brought about therapeutic effects indistinguishable from those of vehicle-treated T cells (CELLS

+ ML-7 in **Fig 4, below**). Tumors in these mice showed the same temporary delay in growth and eventual relapse as those of mice infused with vehicle-treated T cells.

There are several possible explanations for the lack of effect. One is that is that the increased localization was offset by a long lasting depression of T cell function. We have shown that both proliferative activty and cytotoxicty quickly returns to normal levels in T cell populations given the same transient ML-7 treatment used in this study (Task 2a,b, below). Migration of activated T cells into extracellular matrix *in vitro* also recovered quickly after ML-7 treatment. The treated cells also appeared to be unimpaired in their ability to extravasate into tissue *in vivo*. This was apparent from the positions of labeled T cells in frozen sections of tumors (**Fig. 4D,E of Appendix. Reproduced as Fig. 3D,E, above**). It is still possible that T cell cytokine secretion is necessary for antitumor effect in this system, and that this secretion was persistently impaired by ML-7 treatment. This possibility could not be tested during the project period and will be reserved for future work. Finally, it is possible that even the fourfold increase in tumor localization produced by ML-7 pretreatment is not enough to make a difference in therapeutic effect. Even with ML-7-mediated increase, only about 2% of the total cellular input reached the tumors. It is therefore important to continue to develop new and better strategies for improving the survival and tumor-homing of adoptively transferred T cells. Also worthy of consideration in future work is the possibility that the modest increase in localization attainable by ML-7 treatment may be therapeutically important when lymphocytes are used as vehicles of gene therapy rather than direct killers of tumor cells [8;9]. All of these possibilities will be tested in futire work.

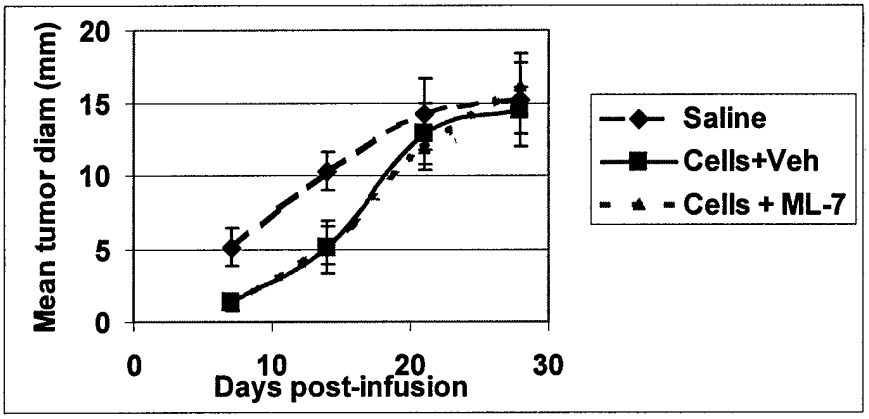

Fig. 4 . Effects of of ML-7-mediated transient depolarization of ErbB2-specific T cells upon their immunotherapeutic effect vs. D2F2 mammary tumor. Mice bearing bilateral ErbB2-expressing D2F2/E2 mammary tumors were given a single tail-vein infusion of anti-ErbB2 T cells stimulated in vitro. Infusions were given on day 14 after tumor implantation, which corresponds to day zero post-infusion of the X axis. "Saline" = HBSS infusion only. "Cells + Veh" =, T cells treated with DMSO vehicle 20 min, washed 3X, infused into tail vein in 0.5 ml HBSS. "Cells + ML-7" = T cells treated with 75 uM ML-7, washed 3X, and infused in HBSS. Values represent mean of a total of 6 mice (12 tumors) per condition.

Aim 2 To determine the effects of transient depolarization on immunotherapeutically important properties of antigen-specific T cells, including proliferative, cytolytic and secretorye. This was to be accomplished in

Task 2. "Determine effects of myosin-inhibiting drugs on immunotherapeutically important functions of activated T cells *in vitro"*

TASK 2A . Determine effects of myosin-inhibiting drugs on proliferation in response to antigen stimulation.
TASK 2B. Determine effects of myosin-inhibiting drugs on specific cytotoxicty.

The findings were already summarized in the interpretation of the therapeutic experiment, and in, **Fig. 2., above** Briefly, When ErbB2-specific T cells were treated with ML-7 and washed, they recovered both normal proliferative potential and specific cell-mediated cytotoxicity against D2F2 target cells within 24 hr.

*TASK 2C. **Determine effects of myosin-inhibiting drugs on** lymphokine secretion in response to antigen stimulation.*

Time and resources for this project were exhausted before this task could be completed. It will be reserved for future work. The results would have helped to illuminate the reasons for the main findings of this project, but they would not have altered those findings.

KEY RESEARCH ACCOMPLISHMENTS:

- Short-term inhibition of myosin function was found to reduce the trapping of adoptively transferred T cells in the pulmonary vasculature and to increase their localization in a murine mammary tumor and in lymph nodes.
- Cytotoxic function and proliferative capability of activated T cells quickly recover from the depressive effects of myosin function inhibition.
- These findings validate our hypothesis that the homing of immunotherapeutic T cells to tumors can be improved through manipulation of the T cell cytoskeleton. This is the first time such an effect has been demonstrated

REPORTABLE OUTCOMES:

The products of the first year of work were reported at the Era of Hope Meeting, Orlando FL, September 2002 (poster P22-19).

Manuscript submitted to Cellular Immunology (and attached as Appendix):

Ratner, S., Wei, W.Z., Oliver, J, and Oliver, J. Enhancement of T cell localization in mammary tumors through transient inhibition of Tcell myosin function.

PERSONNEL RECEIVING PAY FROM THIS PROJECT:

Stuart Ratner, Ph.D., Principal Investigator
Jennifer Oliver (nee Moreno), Research Assistant.

CONCLUSIONS:

SUMMARY. The results of the in vivo localization experiments of Task 1 indicate that transient depolarization of activated T cells by a myosin light-chain kinase inhibitor allowed those cells to avoid massive trapping and damage in the pulmonary vasculature after i.v. infusion. This resulted in a significant, fourfold increase in the percentage of infused cells to reach mammary tumors. There was also greater homing to peripheral lymph nodes, where the T cells may recruit additional effectors and establish long term anti-tumor memory. The most likely reason for these favorable changes is that avoidance of pulmonary trapping led to an increase in surviving and undamaged T cells. However, in an immunotherapeutic trial, the improved homing did not benefit the outcome of an adoptive immunotherapy regime in mammary-tumor bearing mice. The results of Task 2 experiments suggest that the lack of effect is not caused by long-term harm to T cell properties such as cytotoxic activity and ability to migrate and to proliferate in response to antigen. Future work should concentrate on combining our cytoskeletal alteration strategies with other developing strategies for improving adoptive immunotherapy, such as transduction of T cells with cytokines which enhance immunotherapeutic effect and T cell survival.

IMPORTANCE. The results constitute the first validation of the concept that direct alterations of the T cell cytoskeleton can improve the trafficking of therapeutic lymphocytes to sites of tumor growth. In this case, the manipulation was a simple transient pharmacological inhibition of function of the motor protein myosin, which is necessary both for the formation of cellular appendages and the maintenance of many types of adhesion receptor in activated configuration. In the future, more specific and controllable cytoskeletal alteration strategies may be developed for pre-infusion treatment of patient T cells. Among the likely possibilities would be antisense RNA against the members of the Rho family of GTPases, which regulate specific features of cellular polarity and motility [10;11].

"SO WHAT?" Adoptive immunotherapy is in theory an ideal way to provide at least short-term immunosurveillance to breast cancer patients whose immune systems have been debilitated by intensive therapy, but in practice the strategy is little used because of its poor track record in clinical trials. Our findings suggest a way to make adoptive immunotherapy more efficient and may therefore lead to a reconsideration of this mode of treatment.

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APPENDIX

MANUSCRIPT SUBMITTED FOR PUBLICATION IN CELLULAR IMMUNOLOGY

ENHANCEMENT OF T CELL LOCALIZATION IN MAMMARY TUMORS THROUGH TRANSIENT INHIBITION OF T CELL MYOSIN FUNCTION

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Keywords: T cell, immunotherapy, breast cancer, traffic, migration, cytoskeleton, actin, myosin

Running title: T cell myosin and tumor infiltration

ABSTRACT

A limitation on the effectiveness of adoptive immunotherapy is the poor survival and tumor localization of activated T cells after infusion. Activated T cells display a persistent polarized and adhesive phenotype. We hypothesize that this phenotype makes the cells prone to embolize and incur damage in pulmonary microvasculature; and that transient inhibition of T cell polarity and adhesiveness, induced just before infusion, will improve the survival and circulatory abilities of activated T cells. A protocol was developed wherein activated T cells are depolarized by brief exposure to the myosin light-chain kinase inhibitor ML-7. The cells regained polarity, adhesiveness and motility by 6 hr post-treatment and recovered cytotoxic and proliferative activity within 24 hr. The effects of ML-7 pretreatment on T cell traffic in vivo was determined in a murine model in which ErbB2-specific T cells were infused i.v. into hosts bearing the syngenic ErbB2-expressing mammary tumor D2F2/E2. ML-7 pretreatment proved to reduce short-term pulmonary trapping by a factor of eight, and increased fourfold the homing of ErbB2-specific T cells to s.c. tumors. There was also a significant increase in the homing of T cells to peripheral lymph nodes. In a therapeutic trial, though, ML-7-enhanced localization did not improve the antitumor effects of T cell infusion. This work is the first proof that cytoskeletal alteration of T cells can improve their trafficking behavior in an immunotherapeutic situation. The results indicate, however, that the strategy will be most useful if combined with other methods of enhancing T cell survival and function.

Introduction

Several modes of immunotherapy show potential promise against established malignancies, though each has particular advantages and drawbacks. Adoptive T cell immunotherapy allows a T cell response against tumor antigens to be generated under optimal conditions, removed from tumor-derived suppressive and tolerogenic influences. and during periods of immunosuppression following radiation and chemotherapy [1]. Apotive transfer therapy also permits the use of promising new genetic manipulations of the T cells during ex vivo culture, such as the expression of specific Ag receptors and of cytokines which enhance antitumor response and T cell survival [2;3]. A way to further improve adoptive immunotherapy is to increase the ability of cultured lymphocytes to traffic and survive efficiently when returned to circulation.. Localization studies in animals and patients have shown that activated T cells, after i.v. infusion, show massive short-term arrest in lung microvasculature. After clearance, they localize heavily in liver, suggesting that they have been damaged or otherwise altered in ways which limit their ability to circulate [4;5]. Only a small percentage of infusate reaches tumor sites or lymph nodes [6]. Even though some transferred cells certainly do show long term engraftment and growth [2;3;7] the effectiveness of adoptive therapy still depends strongly on the infusion of a large number of T cells [8].

We hypothesize that pulmonary arrest stems in large part from the polarized, adhesive phenotype of the cultured activated T cells. T cells normally circulate in a state of rest or early activation, in which they are round, smooth, and nonadhesive. Activation in vitro converts them into a polarized, spiky, adhesive and highly motile form which they never

normally achieve until after extravasation [9] and which, we propose, contributes to their embolization and trapping in pulmonary microvasculature after infusion, when cell density is highest. We also hypothesize that transient inhibition of this polarized phenotype, induced shortly before infusion, will allow the cells to escape trapping and its attendant damage, and to show more normal patterns of traffic. The polarized form of activated T cells is not reversed by removal from culture flasks, for it is maintained independent of adhesion to substrate and persists when cells are kept in suspension. We have also found it impractical to return activated T cells to the nonpolarized state by withdrawing stimulation, for this type of depolarization is accompanied by apoptosis for all but a minority of cells (not shown).

In activated T cells, actin-myosin contractility is programmed to produce appendages and to promote the formation of actived integrin-containing adhesive foci [10]. We now report that that activated T cells can be rendered temporarily nonpolar, devoid of appendages, and nonadhesive in vitro by treatment with pharmacological inhibitors of myosin function, in protocols that allow return of full polarization, motility, and cytotoxic function in vitro. We demonstrate, in a murine mammary tumor model, that pretreatment of activated T cells with myosin inhibitors prior to infusion greatly reduces the initial trapping of the cells in lung microvasculature and increases their later localization in tumor and peripheral lymph nodes. We also determined whether transient myosin inhibition improves outcome in an immunotherapy model.

Methods

Cell lines and reagents. D2F2/E2, a mammary adenocarcinoma which arose from a spontaneous mammary hyperplasia of a BALB/C mouse and which was stably transfected to express full-length human ErbB2, was the kind gift of Dr. W.Z. Wei [11]. Also obtained from Dr. Wei was the antigen presenting cell line 3T3/E2, a syngeneic fibroblastoid cell line engineered to stably express human ErbB2 as well as K(d), IA(d)$\alpha\beta$, and the costimulator B7.1 [12].

The myosin light chain kinase inhibitor ML-7 (Calbiochem, La Jolla, CA) was stored as a sterile 100 mM stock solution in DMSO and diluted just before use. The nonspecific green fluorescent tracking dye CFSE ("Cell Tracker", Molecular Probes, Eugene OR) was stored as a 10 mM solution in DMSO.

*T cell culture.*BALB/c mice received bilateral s.c. flank injections of 5 X 10^6 irradiated D2F2/E2 cells. This was repeated 4 weeks later, and after an additional 4 weeks draining lymph nodes were excised. ErbB2-specific lymphocytes from these nodes were specifically expanded by culture on irradiated 3T3/E2 cells. The T cells were thinned and fed every 2 days with DME containing 10%FCS, 5U IL-2/ml and 10 ng/ml IL-7, and restimulated with irradiated 3T3/E2 every 14-20 days. After two rounds of stimulation, the lymphocyte population reached a stable phenotype of >98% CD3+, of which typically 55-60% were CD4+, and 45-50% CD8+. They were used in experiments from 7 - 10 days after their last stimulation. At this point the cells typically exerted approximately

50% specific cytotoxicity against D2F2E2 at a an E:T ratio of 50:1, 35% at 25:1, and 10% at ratio of 10:1, as determined by chromium release assay (not shown), whereas cytotoxicity vs. the unrelated syngeneic mammary tumor 410.4 was <5% (not shown).

Lymphocyte localization experiments. D2F2/E2 tumors were allowed to grow s.c. in the flanks of syngeneic mice to approx. 10 mm diameter (0.4 - 0.6 g), a process which took 3-4 weeks. ErbB2-specific T cells were labeled by 30-min incubation with 1 uM CFSE, a concentration found in pilot experiments to confer sufficient fluorescent brightness to track the cells for at least 48 hr while not altering their specific cytotoxicity or ability to migrate into collagen matrix in vitro (not shown). After labeling, T cells were treated with ML-7 or DMSO vehicle control and washed as described above, and then infused into D2F2/E2-bearing mice via tail vein, 2 X 10^7 cells/0.5 ml HBSS per mouse. At 30 min, 2 hr, 24 hr, and 48 hr, tissues were harvested, and weighed, to the nearest mg. Single-cell suspensions were prepared as follows. LYMPH NODES (pooled inguinal, axillary, cervical, mesenteric) were pressed through wire mesh. SPLEENS were pressed through wire mesh and the resulting suspension was enriched for viable lymphoid cells by centrifugation over Ficoll-Hypaque cushions. LIVERS were minced with a scalpel and incubated at 37°C with constant shaking in an enzyme cocktail until a uniform suspension was obtained (usually 2-3 hr). The cocktail consisted of Liberase Blendzyme 2 collagenase mixture (Roche), 1 U/ml; elastase II (Roche), 2 U/ml, DNAse (Sigma), 3 KU/ml, and 2% CS, all in phosphate-buffered saline (PBS). The resulting cell suspension was filtered through a 60 uM mesh Nitex screen. TUMORS were treated like livers but in addition were centrifuged over Ficoll-Hypaque to deplete nonviable cells. It was

recognized that these dispersal steps caused the loss of an unknown percentage of labeled cells, but there was no reason to believe that the losses were not random. It was therefore assumed that the losses did not bias the comparisons of localization of treated vs. control T cells within each tissue type.

The single -cell suspensions were fixed in 3% paraformaldehyde, washed, and resuspended in a known volume of PBS. A measured aliquot, usually 600 ul, was analyzed by flow cytometry (Becton-Dickinson FACSCalibur). Data were collected through forward and side-scatter gates which excluded nonviable lymphoid cells. In two-dimensional dot plots (forward scatter vs. green fluorescence), labeled T cells appeared in a discrete region which was clearly separated from all unlabeled cells by at least a log of green fluorescence level (e.g. region "R1" in Fig 3A). This region was determined to be empty when only unlabeled cells were infused (not shown). The number of labeled cells in the sample was determined by the analysis function of WinMIDI cytometric software. The number of labeled cells recovered per gram of original tissue was calculated as follows.

$$\frac{(\#\text{ labeled cells per 600 ul cell suspension}) \times (\text{total volume cell suspension})}{(\text{tissue weight in g})} = (\#\text{ labeled cells per g tissue})$$

In some cases tissues were not disrupted but embedded in molds filled with Cryoform (IEC, Needham MA), immediately frozen in isopentane chilled with liquid N2, and stored for frozen sectioning. For visualization of CFSE stained cells, sections were cut at 10 um, air dried, mounted in SlowFade (Molecular Probes) and viewed with an Olympus BX40 microscope equipped with epifluorescence and an MCID image recording and

analysis system (Imaging Research, St. Catherine's, Ontario); or with a Zeiss LSM-310 confocal microscope (Confocal Microscopy Core Facility, Karmanos Cancer Institute).

Measures of lymphocyte function. To measure T cell adhesion to fibronectin-coated surfaces, acid-washed cover slips were placed in 35 mm diam. culture wells,and coated with bovine or human serum fibronectin (Calbiochem), at 50 ug/ml in 50 mM Tris Hcl pH 8.0. Slips were washed and blocked with medium containing 1% BSA, T cells were added at a density of 3 X 10^6 per well in the same medium. After 1 hr, slips were removed from wells, washed gently in PBS, and fixed in 3% paraformaldehyde. Randomly selected 32X microscopic fields were photographed and the number of cells per field quantitated, Results are mean counts in 6 microscopic fields in each of 3 slips per experimental condition.

A collagen gel migration assay was used as a measure of spontaneous T cell motility. An acid solution of rat tail collagen was mixed with ice-cold concentrated RPMI 1640 medium to yield a 1.2 mg/ml collagen monomer solution of physiological osmolality. This was pipeted into 22mm diameter culture wells and allowed to polymerize at 37°C into gels approximately 3 mm thick. Cells were suspended in medium containing 0.05% lipid-free bovine serum albumin (Sigma) and added to the wells at a density of 2 X 10^6 cells per cm^2 gel surface. After a migration period of 12 hr at 37°C gels were fixed by addition of 33% paraformaledhyde to a final concentration of 3%. Leading-front distance was measured as the maximum depth at which $\geq$3 cells were simultaneously in

focus in a 400X field, as determined by a fine-focus adjustment which was calibrated in μm of travel. Triplicate measurements were made each counting cells in 5 fields per gel. Fields were chosen randomly within a 10 mm circle whose center coincided with the center of the gel.

To measure proliferation, ErbB2-specific T cells, cultured as described above were harvested 2 weeks after their last stimulation, when proliferation had essentially ceased. Aliquots of 10^6 cells were treated 20 min with either 75 uM ML-7 or with 0.001% DMSO vehicle control. The cells were then washed and placed on irradiated 3T3/E2 stimulator cells and cultured as above. Cell countes were made during the ensuing 5 days This experiment was repeated three times with different batches of T cells.

The cytotoxic activity of T cells, cells of target line D2F2/E2, were labeled with $Na^{51}CrO4$ (Perkin Elmer Life Sciences), washed throroughly, and dispensed into 96-well round-bottomed plates, at 10^4 cells per well. T cells were tested on days 7 - 12 after their last restimulation. They were divided into two groups, one receiving a 20 min treatment with 75 uM ML-7 and one receiving DMSO vehicle. After three washes, the T cells were returned to culture. In order to measure the effects of ML-7 both immediately after treatment and after various recovery periods, pairs of treated and control cultures were prepared 24 hr, 6 hr, and 0 hr before assay. The T cells were then washed and dispensed into wells at effector to target ratios of ranging from 12.5:1 to 50:1. These will be referred to as "test wells". After 4 hr, a 50 ul sample of supernatant was withdrawn from each well, mixed with 150 ul Optiphase Supermix water-soluble scintillation cocktail,

and released isotope was quantitated by beta counting (Trilux MicroBeta Scintillation Counter), with results expressed as cpm. Spontaneous isotope release was determined from supernatants from target wells containing medium only (to be referred to as "medium" wells), and total incorporated isotope was determined from wells in which targets were lysed with 1% Triton X-100 (to be referred to as "total" wells). Three to five replicate wells were run for each condition, and the experiment was repeated three times. Specific cytotoxicity was calculated as follows:

$$\% \text{ specific lysis} = 100 \text{ X } (cpm_{test} - cpm_{medium}) / (cpm_{total} - cpm_{medium})$$

Statistical analyses of lymphocyte localization and function were performed by two-tailed t tests.

Immunotherapy experiments. D2F2/E2 tumors were initiated s.c. in the flanks of mice, bilaterally, as described above, but were allowed to grow only for 14 days before the start of an experiment, at which point they had become barely palpable. In pilot studies it was determined that this procedure provided a mass of tumor cells large enough for T cells to infiltrate, but small enough to respond to therapy (not shown). In experiments determining the effects of ML-7 on therapy. Tumor hosts were divided randomly into three groups: "CONTROL" mice received a tail-vein i.v. infusion of 0.5 ml HBSS. "CELLS + VEH" mice received 0.5 ml HBSS containing 3 X 10^7 T cells which had been treated with vehicle (0.01% DMSO) for 20 min, washed 3X, and suspended in HBSS.

"CELLS + ML-7" mice received 3 X 10^7T cells which had been treated 20 min with 75 uM ML-7, washed three times and resuspended in HBSS. Beginning at 7 days post-treatment, tumors were measured with calipers at weekly intervals. Two perpendicular measurements of diameter were made and the average value for each tumor will be referred to as "mean tumor diameter" [11]. Three experiments were performed, with 2 mice in each experimental condition in each experiment. ANOVA (Instat, Graphpad) was used to detect significant treatment-induced differences in mean tumor diameter. When differences were found, ANOVA was followed by paired t test with Bonnferoni correction for multiple comparisons.

Results

Transient depolarizing effects of ML-7 treatment.

Anti-D2F2/E2 T cells, after culture to peak numbers and cytotoxic function, displayed the polarized morphology typical of activated T cells, including a broad anterior bearing lamellipodia and a stalk-posterior uropod. The cells tended to clump, especially at the uropodia, which bear concentrations of adhesion molecules and ligands [13-15] (Fig 1A). A 20 min treatment with 75 uM ML-7, followed by three washes, caused 100% of cells to lose polarity and all appendages for 30 - 45 min at 37°C (Fig 1B). Approximately 50% of cells repolarized by 2 hr (not shown) and polarity was fully restored by 6 hr (Fig 1C). Another typical behavior of activated T cells, integrin-mediated adhesion to fibronectin, was also temporarily abolished by ML-7. The time course of adhesion recovery paralleled that of repolarization, and adhesion had returned to control values by 6 hr after treatment (Fig. 1D). We found previously that this cell population binds fibronectin mainly via integrins a4b1 and a5 b1 (not shown); and it has been established that MLCK activity can be required for the maintenance of functional adhesion complexes [10;16]. Adhesion and migration through Type I collagen gel, which was previously found to be mediated principally by a2b1 integrin in these cells, showed a similar transient inhibition after ML-7 treatment (Fig. 1E, 16 hr migration assays). The ML-7 treatment did not appear to reduce lymphocyte viability after i.v. infusion. Fresh resting lymph node cells treated with 75 uM ML-7 homed to lymph nodes at approximately the same rate as did vehicle-treated controls when measured at 24 hr post-infusion (not shown). Treatment of

the T cells with ML-7 concentrations of 50 uM or more did yield significantly longer periods of depolarization with eventual return of polarity and adhesion, but the cells showed impaired lymph node homing and higher liver accumulation after i.v. infusion, suggesting that survival was reduced, perhaps because of excessive fragility (not shown). An ML-7 concentration of 75 uM was therefore chosen as an inducer of transient T cell polarization for the rest of the study.

Effects of myosin-inhibiting drugs on immunotherapeutically important functions of activated T cells *in vitro*

Proliferation in response to antigen stimulation. Two weeks after their last stimulation, when proliferation had essentially ceased, groups of 10^6 ErbB2-specific T cells were treated 20 min with either 75 uM ML-7 or with 0.01% DMSO vehicle control. The cells were then washed and placed on irradiated 3T3/E2 cells stimulator cells and culture continued. There was no significant difference in cell proliferation at any time point (Fig 2A).

Specific cytotoxicty. On days 7 - 12 after their last stimulation ErbB2-specific T cells were divided into two groups, one receiving a 20 min ML-7 treatment and one receiving DMSO vehicle. Their cytotoxicity vs. D2F2/E2 cells was measured in 51Cr release assays after recovery periods of 0 hr, 6 hr, and 24 hr. Assays were performed at a range of E:T ratios. Results for 25:1 are presented in Fig. 2B.

When assays began at 0 hr. after ML-7 treatment, cytotoxicity against D2F2E2 was only 22% that of control T cells (Fig 2B). At 6 hr post-treatment, the ML-7 treated cells had recovered to 42% the cytotoxicity of controls, and by 24 hr there was no longer a significant difference. These results indicate that transient T cell depolarization by 75 uM ML-7 produces no lasting changes on two important surrogate measures of T cell immunotherapeutic function.

Alteration of traffic by pretreatment of T cells with myosin inhibitor.

Decreased lung localization by treated cells. When control cells were infused, their mean 30 min localization in the lungs was 2.1 X 10^5 cells/g ($\pm$ 0.57 X 10^5). In contrast, the treated cells showed more than an eight-fold decrease in localization ($p < 0.05$) (see Fig. 3A,B for example of cytometric analysis, and Fig 3C. for summary). At 2 hr, about 75% of the control cells had left the lung, but ML-7-treated cells still showed a significant (3-fold) lower localization (Fig. 3C). By 24 hr, virtually no consistently measurable population of labeled cells was recovered from lungs. (not shown).

Examination of frozen sections showed that the heavy 30 min localization of infused control-treated T cells in the lung represented not extravasation but trapping within the

vasculature (not shown). The observed kinetics -- prompt accumulation and rapid clearance -- are also typical of such trapping (e.g. [17]). The most likely interpretation of our results is that the round and nonadhesive ML-7-treated cells slipped more easily through the lung microvasculature than did the spiky and adhesive control cells. The alternate interpretation, that the ML-7-treated cells were more fragile than the controls and simply ruptured during passage through the lung, is rendered unlikely by the increased localization of ML-7-treated T cells in tumor and lymph nodes, below.

Increased lymph node localization of treated cells. Activated T cell cultures usually contain a subpopulation of cells capable of homing to peripheral lymph nodes. Mean 24 hr localization of labeled control T cells in a set of peripheral lymph nodes was 5.4×10^5 cells/g ($\pm$ 0.8×10^5). ML-7 treatment increased this localization slightly but significantly, to 7.9×10^5 cells/g ($\pm$ $.0.8 \times 10^5$), an increase of about 1.5 fold. The levels stayed approximately the same at 48 hr (Fig 3D). This difference in homing could not be attributed to a change in expression of CD62L, which was found to be unchanged by ML-7 treatment (not shown).

Increased tumor localization of treated cells. No measurable populations of labeled cells were recovered from s.c. D2F2 tumors at 0.5 or 2 hr, but at 24 hr, mean localization of control T cells was 3.5×10^4 cells/g ($\pm$ 0.9×10^4). . This represents approximately 0.5% of infused cells per tumor and is generally in line withwhat has been reported for other adoptive transfer experiments. ML-7 treated cells showed a greater than 4-fold

increase in tumor localization, a mean of 1.5 X 10^5 cells/g ($\pm$ .47 X 10^5) ($p < 0.05$) (For examples see Figs. 4A and B and summary Fig. 4C). At 48 hr, the longest period attempted, localization of control cells increased slightly, to 5.5 X 10^4 cells/g ($\pm$ 2.0 X 10^5), and treated cells still demonstrated an approximately 4-fold increase ($p < 0.05$) (Fig 4C). In sectioned tumors, fluorescently labelled cells of lymphoid morphology were distributed almost exclusively in tumor parenchyma. This proves that cytometric localization was measuring true extravasation of lableled cells rather than artifacts such as vascular trapping or ingestion of labelled debris by tissue macrophages (Fig. 4D). The most likely interpretation of the results is that transiently depolarized T cells passed through lung microvasculature with more success and less damage than polarized T cells; and that this successful passage resulted in a larger pool of cells available to localize in tumor.

Other localizations. ML-7 pretreatment made no significant difference in the percentage of T cells which localized in liver and spleen (not shown).

Immunotherapeutic effects of transiently depolarized T cells.

In a pilot study, variable numbers of ErbB2-specific T cells were infused i.v. into mice bearing s.c. tumors of the ErbB2-positive mammary adenocarcinoma line D2F2/E2. tumor-bearing mice. Single infusions were given on Day 14 after implantation. Maximal therapeutic effect was achieved with 3 X 10^7 cells and did not increase significantly when 4 or 5 X 10^7 cells were administered. This maximal effect was a significant but temporary reduction in tumor growth. Relative to controls, infusion of T cells reduced

tumor size by a mean of 59% at 7 days, and 42% at 14 days. Differences became insignificant at 21 and 28 days.

This protocol, with its incomplete and temporary reduction in tumor development, was selected as an excellent system for detecting therapeutic improvement brought about by ML-7 treatment of T cells before adoptive transfer. Mice were divided randomly into three groups: "SALINE" control mice received a tail-vein i.v. infusion of 0.5 ml HBSS. "CELLS + VEH" mice received 0.5 ml HBSS containing T cells which had been treated with vehicle (DMSO) for 20 min, washed 3 times, and suspended in HBSS. "CELLS + ML-7" mice received T cells which had been treated with 75 uM ML-7 for 20 min and similarly washed and resuspended.

As expected from the pilot study, a single infusion of T cells pretreated only with DMSO significantly reduced the growth of s.c. D2F2/E2 tumors for the first two weeks after treatment, relative to infusion of HBSS only (SALINE vs. CELLS + VEH in Fig. 5.) Mean reductions were 73% at 7 days and 51% at 14 days. Also consistent with the pilot study, cell infusion had no significant effect on longer-term outcome. By 4 weeks post treatment all tumors had grown to a size that required euthenasia of host mice in both CONTROL and CELLS + VEH conditions (Fig. 5). .

Despite their greater tumor localization, the ML-7-pretreated cells brought about therapeutic effects not significantly different from those of vehicle-treated T cells (CELLS + ML-7 in Fig 5). Tumors in these mice showed a temporary delay in growth and eventual relapse similar to those of mice infused with vehicle-treated T cells.

The results show that, although transiently depolarized localized more efficiently in D2F2 /E2model tumors and lymph nodes, this altered localization was not in itself sufficient to improve outcome in this immunotherapy model.

Discussion

Several new strategies are being developed to increase the survival and eventual tumor localization of adoptively transferred T and NK cells in tumor immunotherapy. When feasible, the cells can be infused directly into the arterial supply of an affected tissue. This gives the lymphocytes an opportunity to localize before facing the challenge of passage through pulmonary vaculature [18;19]. Selection or guided maturation of T cells of effector/memory phenotype can produce populations woth greater tendency to infiltrate inflammatory lesions, including tumors [20;21]. Transducing the cells to overexpress viability-enhancing cytokines, telomerase, and anti-apoptotic proteins can also improve long term survival of transferred T cells [2;3;22]. In this paper we have provided the first validation of a simple strategy which can easily be combined with the others, or used alone: transient reduction in T cell polarity and adhesiveness through pharmacological alteration of the cytoskeleton. In this case, this alteration was brought about by transient T cell depolarization through pretreatment with ML-7, a reversible inhibitor of MLCK. ML-7-treated cells avoided massive trapping in the pulmonary vasculature after i.v. infusion and showed a fourfold increase in the localization in D2F2/E2 mammary tumors. There was also greater homing to peripheral lymph nodes,

where the T cells may recruit additional effectors and establish long term anti-tumor memory.

After activation and culture *in vitro*, T cells and assume a polarized intrinsically motile form. Features of this form include highly activated adhesion complexes which are especially concentrated on the uropod, the posterior stalk-like, relatively rigid appendage which contains the entire microtubule and intermediate-filament array of the cell [9;13]. Adhesiveness and uropod formation are adaptations which aid in the early steps of extravasation, and they normally develop only after lymphocyte arrest on vascular walls [23]. We have hypothesized that aggregation of adoptively transferred T cells via their stiff, adhesive uropodia is a major contributor to their lodging in pulmonary microvasculature after therapeutic infusion.. The current finding, that ML-7-mediated depolarization reduces pulmonary trapping, is consistent with this hypothesis.

The effects of ML-7 are probably attributable to disassembly and/or relaxation of actin-myosin complexes due to the drug's selective inhibition of MLCK. It is well established that phosphorylation of myosin light chains is necessary for the association of myosin with actin and for actin-myosin contractility and motility [24]. Actin-myosin function, in turn, is required for the formation of lamellipodia and filopodia, and the formation and proper spacing of functional adhesive focal contact structures [10;16;25;26]. The consequences of MLCK inhibition in several types of polarized, adhesive cell is to cause rounding, loss of lamellipodia and filopodia, and reduced integrin function [27;28]. These are the effects that we observed when the MLCK inhibitor ML-7 were applied to antigen-activated, cultured murine T cells in this study. The concentration of ML-7, 75

uM, was selected because it was nontoxic and caused only transient inhibition of T cell appendage formation, adhesiveness, proliferation, and cytotoxicty. At 75 uM, ML-7 begins to have inhibitory effects on protein kinase C (PKC) [10;29]. PKC inhibition can affect T cell morphology and adhesiveness in ways resembling inhibition of MLCK (REFS). We cannot rule out the possibility that PKC inhibition made some contribution to the rounding and loss of adhesion of the T cells. Even if this were the case, it does not invalidate the main point of this study, that transient depolarization improves the ability of adoptively transferred cells to circulate and home.

The mechanisms which explain the improved trafficking cannot be precisely determined in this study. The most likely explanation supports our original hypotheses, that pulmonary arrest is caused largely by the polarized, adhesive phenotype of activated T cells; and that transient depolarization allows the cells to escape arrest and its attendant damage to circulatory ability.

ML-7 pretreatment may have allowed the cells to slip easily through pulmonary microvasculature soon after infusion, when they were at high density and the risk of embolization was greatest. This would account for the great reduction in prompt lung localization. By the time cells repolarized, they circulated at low density and no were longer subject to clumping in microvasculature. This would explain why lung localization did not spike at later time points. Avoidance of pulmonary trapping would then have allowed a larger percentage of infused cells undamaged or otherwise altered and thus able to circulate normally during the next 48 hr. This is the most likely explanation of the significant increase in both lymph node homing and tumor localization by ML-7 treated cells. There is no evidence that ML-7 directly altered T cell phenotype

in a way which would have augmented homing. The percentage of cells expressing L-selectin and LFA-1 were not changed after ML-7 treatment in vitro (not shown). We cannot rule out the possibility that other molecules important in homing, such as CCR7, were altered in expression; this possibility is worthy of further study. The improved trafficking ability of the cells may not reflect increased viability because there was no significant decrease in liver localization. It is possible that pulmonary trapping induces more subtle changes in T cells, such as the induction or activation of adhesion molecules inappropriate for circulation. This possibility too is worth testing in the future.

An trivial alternative possibility is that the ML-7 treated cells were fragile and were quickly destroyed, and that the fluorescent cells detected by flow cytometry were merely macrophages which had ingested labeled debris. This might indeed explain the reduction in prompt lung localization but it is ruled out by histological sections of the tumors which clearly showed fluorescence to reside in intact, extravasated cells (Fig 4E). Some of these cells even showed the morphology of polarized lymphocytes, indicating that the infused cells has indeed repolarized and regained migratory ability *in vivo* as they had been observed to do *in vitro* (Fig 1A-C, E).

In an immunotherapeutic trial, the improved homing of ML-7-treated cells did not improve the outcome of an adoptive immunotherapy regime in mammary-tumor bearing mice. It is unlikely that the lack of benefit was caused by severe impairment of immunotherapeutic function by ML-7 treatment. In vitro experiments demonstrated that the degree of myosin inhibition sufficient to alter T cell traffic causes only a brief decline in cytotoxic activity and ability to proliferate in response to antigen (Fig. 2A,B).

Furthermore, the ML-7 treated cells did exert an immunotherapeutic effect, albeit not one different from that of control cells. It cannot be ruled out that other immunotherapeutic functions, such as cytokine secretion may have been depressed persistently by ML-7 treatment. On the other hand, it may be that a fourfold increase in tumor localization is simply not sufficient in itself to have a lasting effect on immunotherapy. This increase still brought less than 2% of infused cells into the microenvironment of each of our experimental tumors. Transient depolarization may prove to be most useful when combined with other compatible immunotherapeutic strategies such as such genetic augmentation of T-cell function and survival.

Acknowledgments

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Figures

Figure 1. Effects of 20-min. treatment with MLCK inhibitor ML-7 (75 uM) upon morphology, adhesion, and motility of activated anti -D2F2/E2 T cells. **A-C.** Alterations in morphology. **A.** Prior to treatment. Note polarized morphology and frequent cell aggregates. **B.** Thirty min. after treatment and drug wash-out. Note smooth, spherical morphology and lack of aggregation. **C.** Six hours after drug wash out. Bar = 10 um. **D.** Changes in ability of anti-D2F2/E2 T cells to adhere to fibronectin over a period of 1 hour. **E.** Changes in spontaneous 16-hr. migration into three-dimensional gels of Type I collagen. In **D.** and **E.**, X-axis indicates number of hours elapsed between drug wash-out and start of assay and asterisks indicate significant differences between control and ML-7 values ($p<0.05$).

Figure 2. Recovery of anti-D2F2/E2 T cell function after transient depolarization by ML-7. A. Proliferation during incubation with irradiated stimulator cells. B. Cytotoxicity vs. target tumor cells at E:T = 25:1. Asterisks = significantly different from control $p < 0.05$.

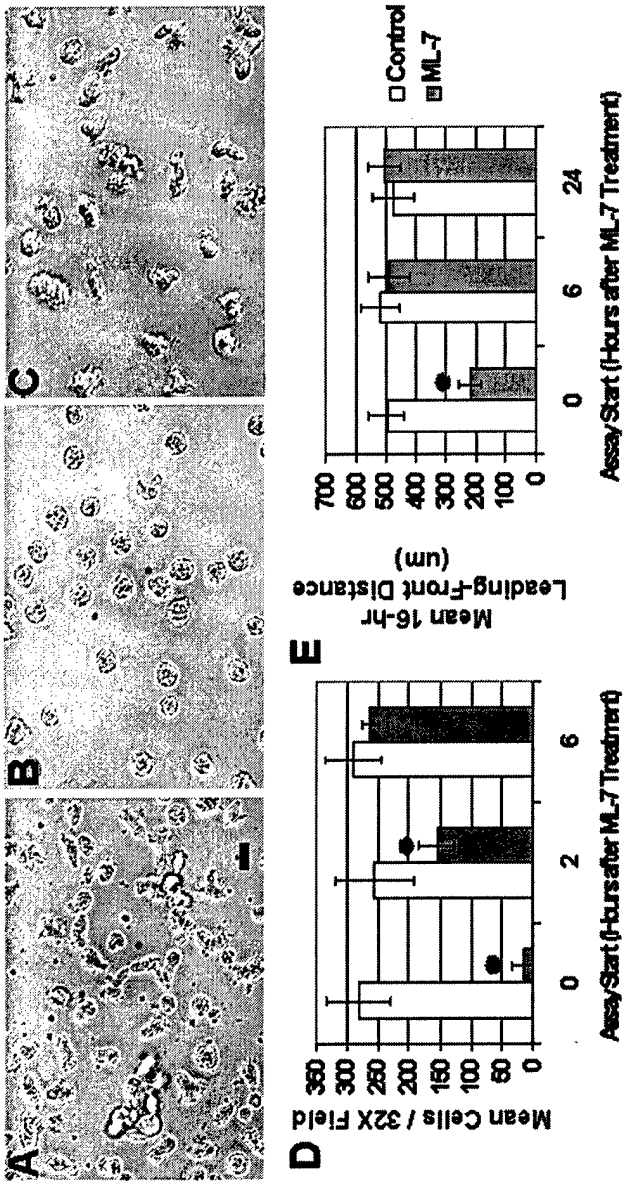
Figure 3. Alteration of T cell localization in lungs and peripheral lymph nodes after ML-7 treatment. **A-C.** Localization of CFSE-labeled T cells in lungs at 0.5 and 2 hr. post-infusion. **A and B.** Typical flow cytometric analyses of single-cell suspensions of lungs at 0.5 hr after infusion of 2×10^7 labeled cells. **A.** Control-treated T cells. **B.** T cells treated 20 min with 75 uM ML-7. R1 = region of high fluorescence determined to be

unique to viable labeled T cells. **C.** Data summary from entire series of lung localization experiments. **D.** Localization of labeled cells in pooled sets of peripheral lymph nodes. . Asterisks = significantly different from control $p< 0.05$.

Figure 4. Localization of CFSE-labeled T cells in s.c. D2F2/E2 tumors. **A and B.** Typical flow cytometric analyses of single-cell suspensions of tumor at 24 hr after infusion of 2×10^7 labeled cells. **A.** Control-treated T cells. **B.** T cells treated 20 min with 75 uM ML-7. R1 = region of high fluorescence determined to be unique to viable labeled T cells. **C.** Data summary from entire series of experiments. Asterisks = significantly different from control $p< 0.05$. **D. and E.** Typical frozen section of tumor showing that fluorescent events detected by flow cytometry represent intact CFSE-labeled cells which have extravasated into tumor parenchyma. **D.** Phase contrast image. **E.** Green fluorescence image of same section. Tumor parenchyma is still visible by faint autofluorescence. Bar = 100 uM.

Figure 5. . Effects of of ML-7-mediated transient depolarization of ErbB2-specific T cells upon their immunotherapeutic effect vs. D2F2 mammary tumor. Mice bearing bilateral ErbB2-expressing D2F2/E2 mammary tumors were given a single tail-vein infusion of anti-ErbB2 T cells stimulated in vitro. Infusions were given on day 14 after tumor implantation, which corresponds to day zero post-infusion of the X axis. "Saline" = cell-free HBSS infusion only. "Cells + Veh" = T cells treated with DMSO vehicle 20 min, washed 3X, infused into tail vein in 0.5 ml HBSS. "Cells + ML-7" = T cells treated

with 75 uM ML-7 20 min., washed 3X, and infused in HBSS. Values represent mean of a total of 6 mice (12 tumors) per condition.


A
B
C
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Mean Cells / 32X Field
Assay Start (Hours after ML-7 Treatment)
Mean 16-hr Leading-Front Distance (um)
Assay Start (Hours after ML-7 Treatment)
Control
ML-7

Fig. 1

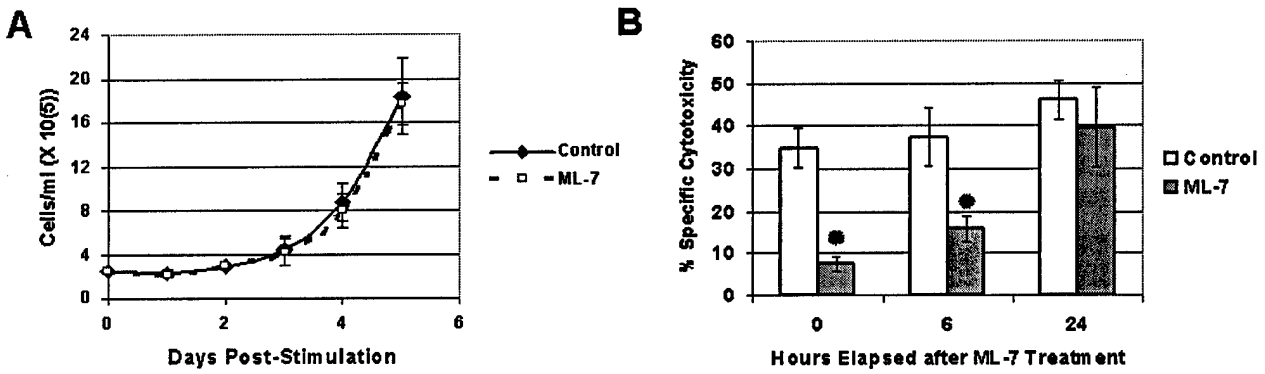

A
Cells/ml (X 10(5))
24
20
16
12
8
4
0
0
2
4
6
Days Post-Stimulation
Control
ML-7
B
% Specific Cytotoxicity
60
50
40
30
20
10
0
0
6
24
Hours Elapsed after ML-7 Treatment
Control
ML-7

Fig. 2

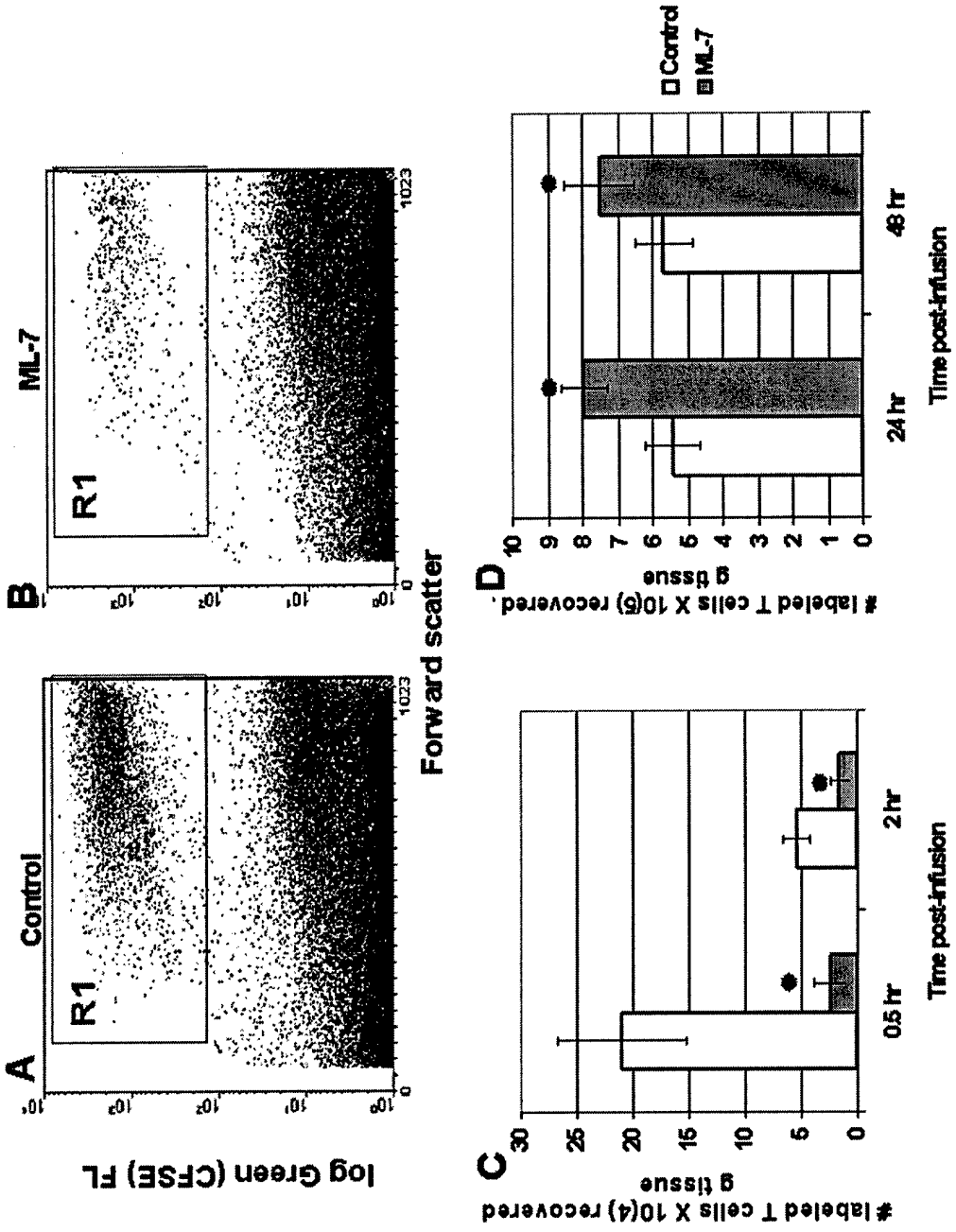
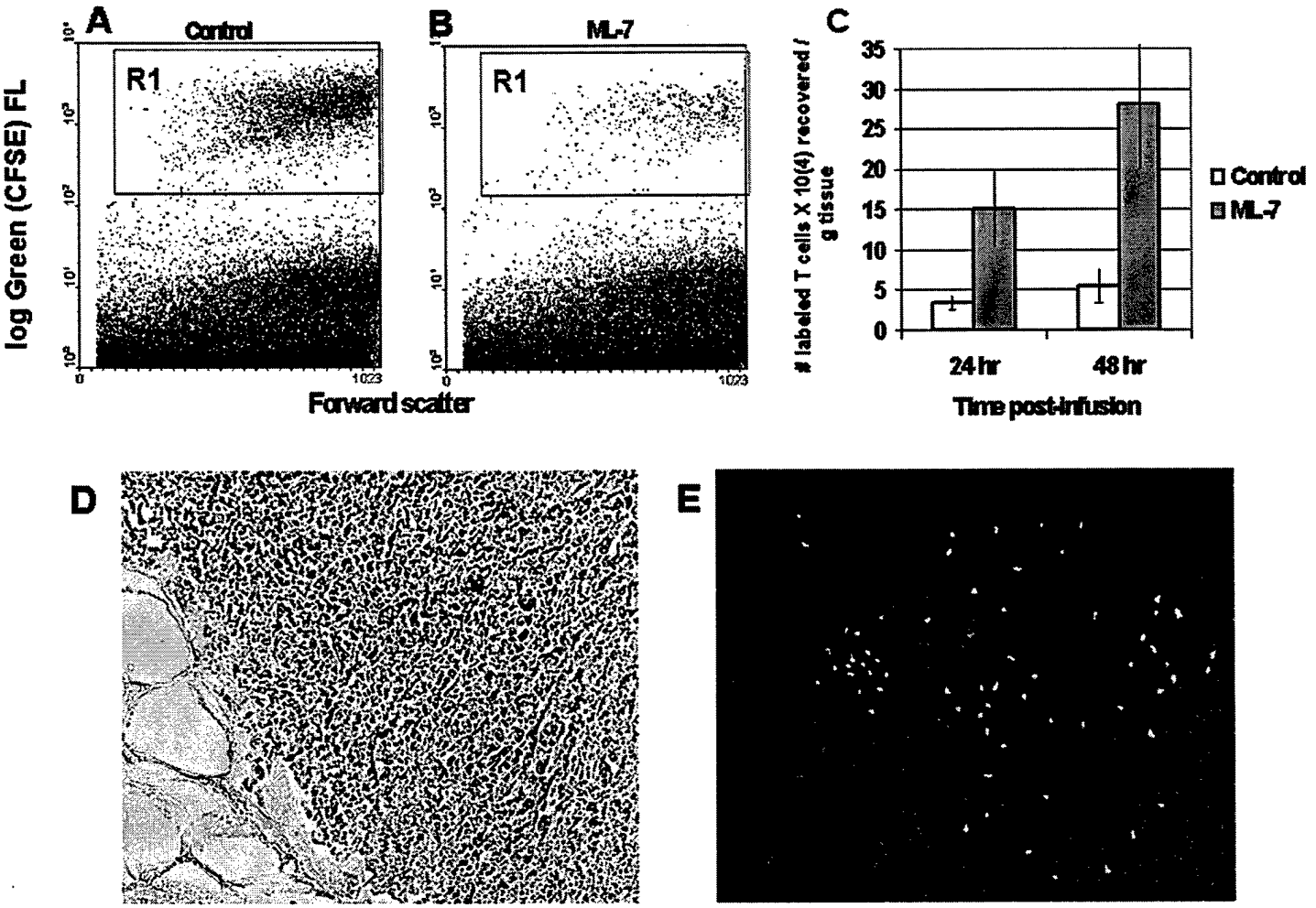

A
Control
R1
B
ML-7
R1
Forward scatter
log Green (CFSE) FL
C
labeled T cells X 10(4) recovered
g tissue
0.5 hr
2 hr
Time post-infusion
D
labeled T cells X 10(5) recovered
g tissue
Control
ML-7
24 hr
48 hr
Time post-infusion

Fig. 3

Fig. 4


A
Control
R1
B
ML-7
R1
log Green (CFSE) FL
Forward scatter
C
labeled T cells X 10(4) recovered / g tissue
35
30
25
20
15
10
5
0
24 hr
48 hr
Time post-infusion
Control
ML-7
D
E

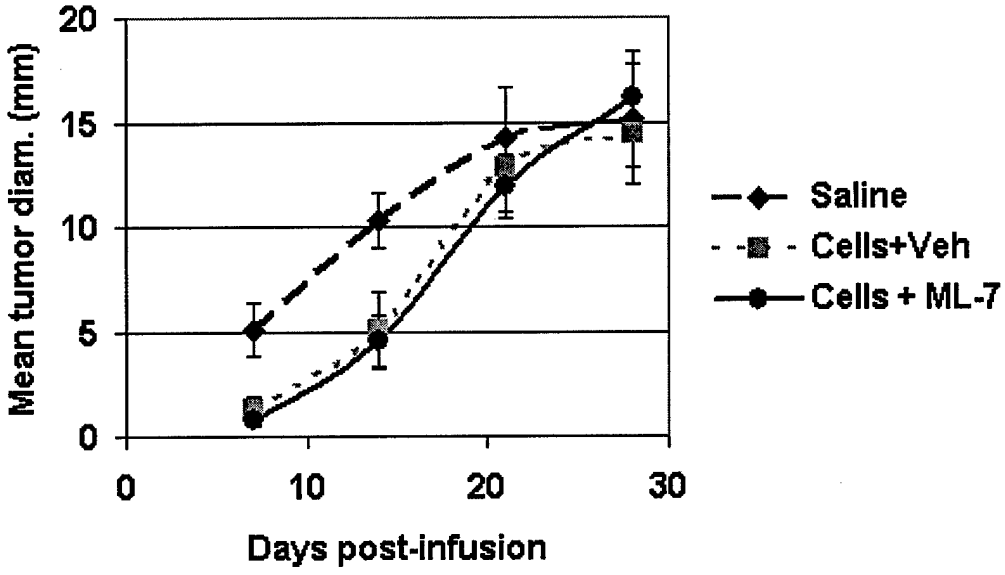

20
15
10
5
0
Mean tumor diam. (mm)
0
10
20
30
Days post-infusion
Saline
Cells+Veh
Cells + ML-7

Fig. 5

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